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13. ABSTRACT (Maximum 200 Words)

In budding yeast, Cdc13 has both an essential function in chromosome end protection as well as a non-essential role in telomere replication, by mediating recruitment of telomerase to the chromosome end. Using fusion protein techniques, we have determined that the essential function of Cdc13 is to deliver an Stn1-containing end protection complex to the telomere. This delivery is sufficient to protect the chromosome, but is unable to provide telomere replication. However, delivery of a small region of Cdc13 is capable of restoring telomerase recruitment in the absence of intact Cdc13 protein. In addition, we performed a genetic screen designed to identify hypothesized processing activities at telomeres. Several interesting mutants that were uncovered have been classified and their role in telomere maintenance is being analyzed. I am presently working in the biochemical characterization of the proteins involved in end protection, and in the identification of metazoan homologs of these proteins. It is our belief that the information derived from the experiments detailed in this proposal may be extrapolated to the corresponding biological situation in humans, where protection of chromosome ends is critical for maintaining genomic integrity and preventing genomic instability.

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INTRODUCTION

Eukaryotes contain specialized structures at their chromosomal termini called telomeres, which form protective caps and help maintain chromosome stability. Telomeres are replicated by the reverse transcriptase telomerase. Although most normal human somatic cells lack telomerase activity (Kim et al., 1994; Broccoli et al., 1995; Counter et al., 1995), 80-90% of human tumors have reactivated the enzyme (Shay and Bacchetti, 1997). Notably, telomerase activity can be detected in most (95%) invasive human breast cancers (Mckenzie et al., 1999). Activation of the telomerase enzyme appears to be necessary for cell immortalization (Kiyono et al., 1998), and is thus a critical step in the development of most cancers (Shay et al., 1993; de Lange, 1994). Since telomerase activation occurs early in breast cancer progression, telomerase may be an important molecular target for breast cancer treatment (Mokbel, 2000). Consistent with this proposal, it has been shown that certain anti-telomerase agents inhibit spontaneous immortalization of human Li-Fraumeni syndrome breast epithelial cells *in vitro*, underscoring the promise of this approach (Herbert et al., 2001). Further research into the potential use of anti-telomerase drugs in breast cancer treatment will require a more detailed understanding of telomerase function and regulation. Many aspects of telomerase regulation in human cells are currently poorly understood: only a limited number of enzyme components have been identified, and little is known about telomerase regulation. However, numerous facets of telomere biology are conserved among all eukaryotes and, consequently, more experimentally tractable model organisms have been employed to facilitate the elucidation of telomerase function at the molecular level – with the eventual goal of applying such knowledge towards understanding comparable mechanisms in humans. This is exemplified by the fact that not only the catalytic core of telomerase is conserved among eukaryotes, since homologs of the Est1 subunit of telomerase have been identified in human cells (Reichenbach et al., 2003; Snow et al., 2003). Proteins that bind to the very extreme of chromosomes, such as Cdc13 in yeasts, have also been described in mammalian cells (Baumann and Cech, 2001), suggesting that research performed in the model organism *Saccharomyces cerevisiae* can be translated into the function of metazoan homologs of our proteins of interest. Our research focuses on determining the mechanisms by which telomeres protect chromosome ends from being treated as DNA breaks and from what activities are telomeres and the proteins that bind them protect. Toward that end we study the roles of Cdc13 and its binding partners in telomere replication and end protection.

RESEARCH SUMMARY (BODY)

Background

Telomeres, the ends of linear chromosomes in eukaryotic organisms, are essential for the maintenance of genomic stability and for DNA replication (Hughes and Lundblad, 1998; Kolodner et al., 2002). In the budding yeast, *Saccharomyces cerevisiae*, telomeres are maintained by the action of several protein complexes that bind to chromosome termini, presumably at different times during the cell cycle. Past research in our lab has focused

on a subset of these complexes. The first complex of interest is the telomerase holoenzyme, which replicates telomeres. This enzymatic complex is composed of a reverse transcriptase subunit (Est2), an RNA subunit (Tlc1), and two regulatory subunits (Est1 and Est3). Another protein complex involved in telomere maintenance is the Stn1/Ten1 complex. This complex, which consists of at least the Stn1 and Ten1 proteins, protects telomeres from degradation (Pennock et al., 2001) and it also inhibits excessive elongation of telomeres by telomerase (Chandra et al., 2001).

Our research goals are to elucidate the mechanisms of chromosome end protection, and telomere replication. One main question regarding chromosome end protection is the role of Cdc13 at telomeres. We addressed this question during the funding period of this proposal and the results are summarized in this report as well as in previous reports. These findings led to a publication in *Cell* (Pennock et al., 2001). In summary, our results led to the proposal that Cdc13 essential function is to recruit an end protection complex, composed of at least Stn1 and Ten1 to telomeres. In the same set of experiments, we also identified the domain by which Cdc13 recruits telomerase. In order to find new genes involved in telomere maintenance, we performed a screen and identified a new class of long telomere mutants, which we attempted to characterize during the funding period of this award. Since we discovered that Cdc13 essential role is to localize Stn1 to chromosome termini, we also want to characterize this protein. Toward that end we are preparing recombinant proteins as well as taking a bioinformatics approach to analyze this essential protein. Lastly, we are interested in finding the activities that process the telomere prior to Cdc13 binding. To this end, we performed a genetic screen that provided us with several classes of mutants, which we are in the process of identifying and characterizing. In this report, I will summarize our previous findings as well as discuss our most recent results in determining the mechanisms of telomere processing and end protection.

Task 1: Determine whether the essential end protection function of Cdc13 is delivery of Stn1 to the telomere

Rationale

Loss of Cdc13 function results in rapid and extensive loss of one strand of the telomere and a subsequent Rad9-mediated cell cycle arrest (Garvik et al., 1995). This ultimately lethal event identifies an essential role for Cdc13 in the protection of chromosome ends from degradation. The mechanism of this protection is unknown, although models suggest that Cdc13 is needed to regulate the processing of the DNA ends by an as yet unidentified exonuclease. Stn1 is an essential protein that exhibits a number of genetic interactions with Cdc13. Loss of Stn1 function also provokes a Rad9-mediated arrest and extensive single-strandedness at the telomere (Grandin et al., 1997). This led us to propose that Stn1 collaborates with Cdc13 at the telomere to effect end protection. The "fusion protein technique" allowed testing the hypothesis that the essential function of Cdc13 is to deliver Stn1 to the telomere, as this model predicts that tethering Stn1 to the telomere would bypass the cell's essential requirement for Cdc13. The results of these

experiments were published in *Cell* in 2001 (Appendix A, Figures 1-2 of 2001 report), and the main results of those experiments are described in the following summary.

Results and Discussion

A. Construct fusions of Stn1 to the DNA-binding domain of Cdc13: The DNA-binding domain of Cdc13 had been previously identified and characterized (Hughes et al., 2000). This subdomain binds single-stranded telomeric DNA with high affinity, but cannot provide the essential function. A construct designed to deliver Stn1 to the telomere in the absence of *CDC13* function was made by fusing the DNA binding domain of Cdc13 (DBD_{CDC13}) to the N-terminus of Stn1. This fusion protein expressed by the *CDC13* promoter on a low copy plasmid.

B. Determine whether fusion proteins are competent for Stn1 function: The fusion protein mentioned above complemented an *stn1*-Δ strain for both viability and telomere length (Figure 1), indicating that the presence of the DBD_{CDC13} had not altered Stn1 activity.

C. Determine if delivery of Stn1 to the telomere provides end protection and allows a cell to grow in the absence of Cdc13: Delivery of Stn1 to the telomere, by fusing Stn1 to the high affinity DNA-binding domain of Cdc13 (DBD_{CDC13}), was sufficient to bypass the essential requirement for Cdc13 in the cell. This data argues that Stn1 can provide an end protection function when delivered to the telomere (Appendix A, Figure 1A 2001 report).

D. Characterize the telomere shortening and senescence phenotypes of a cdc13Δ/pDBD-Stn1 strain: Although overall telomere structure in the *cdc13-Δ/DBD*$_{CDC13}$*-STN1* strain looked normal, telomere length was reduced (Appendix A, Figure 1B 2001 report), suggesting that telomerase recruitment could not occur in the absence of the intact Cdc13 protein. Consistent with this proposal, additional telomere shortening was observed with continued propagation, resulting in eventual senescence (Appendix A, Figure 1C 2001 report). Furthermore, after extended propagation of the *cdc13-Δ/DBD*$_{CDC13}$*-STN1* strain, healthy growing survivors were identified (Appendix A, Figure 1D 2001 report) that exhibited rearrangements of telomeric and sub-telomeric sequences that are characteristic of the survivors recovered from telomerase-defective strains (Lundblad and Blackburn, 1993). The growth defect of the *cdc13-Δ/DBD*$_{CDC13}$*-STN1* strain was further exaggerated by the introduction of a *rad52*$^-$ mutation (data not shown), consistent with previous demonstrations that the appearance of telomerase-defective survivors is blocked by a *rad52*$^-$ mutation (Lundblad and Blackburn, 1993; Lendvay et al., 1996). Therefore, the *cdc13-Δ/DBD*$_{CDC13}$*-STN1* strain exhibits an *est*$^-$ phenotype, based on all of the criteria that have been previously used to characterize telomerase-defective strains.

E. Ascertain whether the telomerase deficiency of the cdc13Δ/pDBD-Stn1 strain can be complemented by delivery of telomerase to the telomere: A DBD_{CDC13}-telomerase fusion was capable of rescuing the telomere replication defect of the *cdc13Δ/DBD*$_{CDC13}$*-STN1* strain. However, telomeres in this strain were elongated greatly when compared to

wild-type. This was reminiscent of the telomere profile observed in a wild-type cell carrying only the DBD_{CDC13}-telomerase fusion, suggesting that the elongation was due to the increase in affinity of telomerase for the telomere (Appendix A, Figure 2 2001 report).

Task 2. Define the recruitment domain of Cdc13

Rationale

The telomerase holoenzyme is required to ensure that sequence is not lost from the termini with each round of replication. In *S. cerevisiae*, the functions of at least five genes are required *in vivo* for telomerase activity: *EST2* and *TLC1* (comprising the enzymatic core), as well as *EST1, EST3,* and *CDC13*. This role for *CDC13* was uncovered through analysis of the *cdc13-2* allele which displays an Est^- phenotype, putting it in the telomerase pathway (Lendvay et al 1996; Nugent et al., 1996). Further work suggests that the defect in *cdc13-2* is an inability to recruit telomerase to the telomere, and this defect can be bypassed by delivering telomerase to the telomere using DBD-telomerase protein fusions (Evans and Lundblad, 1999; Hughes et al., 2000). In the course of the project described in section A of the body of the 2001 report, the region of Cdc13 required to provide its recruitment function was identified. These results have also been published (see Appendix A, Figure 3 2001 report); the main results of those experiments are summarized below.

Results and Discussion

A. Create a series of constructs fusing the DBD of CDC13 to regions of Cdc13 suspected to be involved in recruitment: This experiment relies on the assumption that the telomerase recruitment function resides within a specific domain of the Cdc13 protein. To localize this proposed domain, a set of protein fusions (termed DBD_{CDC13}-RD fusions, where "RD" stands for the telomerase recruitment domain) were constructed that linked the minimal DNA binding domain with the proposed telomerase recruitment region. Eleven nested regions of Cdc13, ranging from amino acids 190 to 340, were fused in frame at the C-terminal boundary of the DNA binding domain (note that this order is the opposite to that found in the native protein; see Appendix A, Figure 3A 2001 report).

B. Determine which of these fusions are capable of complementing a cdc13-2 strain: To test for telomerase recruitment activity, each fusion was first transformed into the recruitment-defective *cdc13-2* strain. Three fusions (DBD_{CDC13}-$RD_{aa190-340}$, DBD_{CDC13}-$RD_{aa211-340}$, and DBD_{CDC13} –$RD_{aa211-331}$, rescued the senescence of the *cdc13-2* strain (Figure 2A). In addition, these three fusions were capable of complementing the telomere length defect of the *cdc13-2* strain (Figure 2C). The comparable mutant fusions, containing the *cdc13-2* missense mutation at amino acid 252 (denoted as DBD_{CDC13}-RD^{est}) were unable to complement *cdc13-2* (Figure 2B).

C. Test whether introduction of the DBD-RD fusion can complement the senescence and telomere shortening phenotypes of a *cdc13Δ/pDBD-STN1* strain: The *DBD $_{CDC13}$-RD* was capable of preventing the telomere shortening and senescence observed in a *cdc13Δ/DBD$_{CDC13}$-STN1* strain. However, unlike with the *DBD$_{CDC13}$-EST1* fusion that greatly elongated telomeres, the telomeres in this strain were maintained at a length that was approximately the same as wild type telomeres. These results collectively demonstrate that wild type telomere length can be reconstituted if both end protection and telomerase recruitment activities can be delivered to the telomere as fusion proteins (Appendix A, Figure 3C-D 2001 report) slightly short but stable. In contrast, the *DBD$_{CDC13}$-RDest* fusion did not prevent the telomere shortening or the growth defect in the *cdc13Δ/DBD$_{CDC13}$-STN1* strain.

D. Characterize the genetic interaction of the RD and Est1 in the context of the fusion proteins: Several experiments were performed that demonstrated that the RD fusion protein retained the same genetic properties as the full length Cdc13 protein with regard to telomerase recruitment. First, introduction of the cdc13-2 mutant allele into the *DBD$_{CDC13}$-RD* fusion results in the loss of the ability of this fusion to promote telomere maintenance. Second, this *DBD$_{CDC13}$-RD* fusion also displayed a genetic interaction with EST1 that re-capitulated the behavior of the full length Cdc13 and Est1 proteins. This second experiment relied on a particular allele of *EST1*, called *est1-60* (isolated by another member of the laboratory, Kathleen Buckley) reciprocal suppression with the *cdc13-2* allele (Appendix A, Figure 4A-C 2001 report). The suppression extends to the recruitment domain as well. Specifically, a *cdc13Δ est1-60/DBD $_{CDC13}$-STN1 DBD $_{CDC13}$-RDest* strain had wild-type telomeres and no apparent growth defect (Appendix A, Figure 4D 2001 report). Therefore, the RD domain retained exactly the same properties as the full length Cdc13 protein with regard to its proposed telomerase recruitment activity.
These reciprocal suppression results provided strong genetic support for a direct interaction between *CDC13* and *EST1*. Further support for this interaction has also been obtained by yeast two hybrid analysis. Using this approach, we observed that Cdc13 interacts physically with Est1, and that the interaction is abolished by the *cdc13-2* mutation (Figure 3).

Task 3. Investigation and characterization of a new class of long telomere mutants

Rationale

Initial studies on Cdc13 indicate that the protein is critical for both telomerase regulation as well as telomeric end protection, but the knowledge of the identity of other proteins also required for these activities remains limited. In order to identify additional proteins that function at the telomere with Cdc13, our laboratory completed a large-scale screen to isolate genes that exhibit a genetic interaction with *CDC13* as well as an altered telomere length profile when mutated. The screen identified 91 mutant strains; 58 strains displayed short telomeres and 33 strains displayed long telomeres. This extensive panel of long telomere mutants represents useful reagents that should allow identification of additional genes that function in parallel with *CDC13* to mediate these functions. Mutations in *TEN1* and *STN1* are expected to be recovered in this screen, since both

genes exhibit a genetic interaction with *CDC13* and mutations in either can confer a long telomere phenotype. The initial goals include characterizing the mutants and the subsequent cloning of the genes containing the causative mutations. Once this is achieved, biochemical and genetic experiments will be completed to determine the function of the newly identified proteins. Such analyses include examining telomeric end structure alterations as well as asking, in a directed manner, which known telomeric proteins may bind these proteins. The analysis of these mutants and the cloning of the responsible genes have the potential to provide information critical to broadening our understanding of telomere biology, and ultimately of breast cancer biology. Information from these studies will be useful for probing the mechanisms underlying telomere deregulation and cellular proliferation in humans.

Results and Discussion

A. Complete initial characterization of the long telomere mutants and determine how many mutants are due to alterations in Stn1 or Ten1: Complementation studies using STN1 present on both high and low copy plasmids demonstrated that 5 of the mutant strains contained mutations in *STN1*. However, 10 strains were not complemented by *STN1*, indicating that at least one additional new gene was identified.

B. Clone the genes responsible for the mutant phenotypes: Several attempts at recovering the new gene(s), by complementation of the mutant phenotype were unsuccessful.

C. Genetic and biochemical analysis of newly-identified genes and *STN1*: Since it has been proposed that Ten1 and Stn1 act as a complex based on the similarity between null and mutant phenotypes for both genes (Grandin et al., 2001), I am performing biochemical characterization of these end protection proteins. So far, I have expressed and partially purified Ten1 as a glutathione S-transferase (GST) fusion protein (Figure 4). I plan to use the purified proteins in telomeric DNA binding assays, and *in vitro* protein binding assays. If good purification is obtained, I also plan to examine the purified proteins for enzymatic activities.

Task 4. Phylogenetic analysis of Stn1

Rationale

To date, very little is known about the role of Stn1 in telomere maintenance, in part due to the relative lack of functionally informative alleles. The original plan consisted of cloning Stn1 homologs from other budding yeasts by using a functional complementation strategy and then use the homologs in multiple sequence alignments to identify conserved residues that might be important for protein function. However, since the sequence of several budding yeasts Stn1 homologs is now publicly available in databases such as the Saccharomyces Genome Database (SGD), the Genome Sequencing Center (GSC) at Washington University, and Genolevures, I have used those as a source instead of cloning Stn1 homologs from other yeasts species.

Results and Discussion

A. Isolate Stn1 homologs from a number of budding yeast species: As mentioned above, this approach was substituted by database searching since DNA and protein sequences of other budding yeasts became publicly available. Using this approach, we obtained the amino acid sequence of species such as *S.castelli*, *S.kudriazevii*, *S.bayanus*, *S.paradoxus*, and *S.kluyveri*.

B. Using alignment tools and sequence analysis programs identify conserved regions of Stn1: An alignment of the Stn1 homologs mentioned above has been produced (Figure 5). This alignment will be used to identify homologs in other yeast species and then identify residues that are important for Stn1 function. This approach is possible because more sequences of other yeasts species such as *K. thermotolerans*, *K. lactis*, and *C. glabrata* have become available for searching for genes of interest. Increasing the number of Stn1 homologs will provide us with a better alignment that will increase our ability to identify important residues for Stn1 function.

C. Design and carry out mutagenesis of Stn1 based on conserved regions identified in part B: The alignments produced in part B will be used to determine which residues of Stn1 will be mutated. Mutagenesis will be performed by site-directed mutagenesis. The mutants will be tested for viability, telomere length, and known interactions with other telomeric proteins. Mutants with interesting phenotypes will be submitted standard genetic and biochemical tests.

Task 5. Identify the hypothesized exonuclease required to process the ends of chromosomes

Rationale

Loss of Cdc13 function results in rapid and extensive loss of one strand of the telomere and a subsequent Rad9-mediated cell cycle arrest (Garvik et al., 1995). This ultimately lethal event identifies an essential role for Cdc13 in the protection of chromosome ends from degradation. The mechanism of this protection is unknown, although models suggest that Cdc13 is needed to regulate the processing of the DNA ends by an as yet unidentified exonuclease.

Several proteins have been implicated in this processing, including Rad24 and Rad50. Rad24 is a component of the DNA-damage checkpoint pathway and part of an RFC like complex that resembles the clamp loading machinery that acts during DNA replication (Shimomura et al., 1998; Green et al., 2000; Naiki et al., 2000). In the absence of Rad24 function, the kinetics of C-strand resection in strains with compromised end protection (*cdc13-1*) were slowed down. These data led to the proposal that Rad24 positively regulates a telomeric processing activity (Lydall and Weinert, 1995; Booth et al., 2001). To test whether Rad24 positively regulated a processing activity, we asked if loss of Rad24 function would allow a *cdc13-Δ* to survive. A *rad24-Δ cdc13-Δ* strain continues dividing until it forms microcolonies, whereas a *cdc13-Δ* strain

arrests at the large-budded stage. The same effects are observed for *stn1-Δ rad24-Δ* and *ten1-Δ rad24-Δ* strains. However, this bypass is only partial, indicating that at least one other enzyme can act at the telomere to promote C-strand resection when Rad24 is absent. If this reasoning is correct, then a genetic screen designed to isolate genes that, when mutated, enhance the growth of *rad24-Δ cdc13-Δ* strains should lead to identification of factors involved in telomeric processing.

Results and Discussion

A. Conduct screens to isolate mutants in a variety of genetic backgrounds that allow for cell growth in the absence of Cdc13: We used transposon-mediated mutagenesis to perform the genetic screen because of a number of reasons, the most paramount being the increased likelihood of creating null alleles using this method. The library used has previously been published (Burns et al., 1994; Seifert et al., 1986). Figure 1 on the 2002 annual report represents a conceptual representation of this screen.

A *cdc13-Δ rad24-Δ/pVL910* (*CEN CDC13 URA3 ADE2*) was subjected to transposon-mediated mutagenesis, and over 35,000 transformants were screened (3.5X coverage of the genome). A total of 761 potential candidates were chosen based on the ability to support growth, of which 544 retested. These 544 were tested to determine which ones would become secondary candidates (see 2002 report for details). After testing, 132 secondary candidates were obtained. A comprehensive list of these secondary candidates can be found in Table 1 of the 2002 report.

B. Identify the mutated gene(s): We identified the site of transposon integration 72 mutant candidates. The criteria used to determine if a candidate was of interest are mentioned in the 2002 annual report. These criteria led to the identification of several classes of mutants. The first class is made of proteins that have a known or suspected role in the ubiquitination pathway. Another class is made of chromatin remodelers. How this mutants affect telomere maintenance is not known as it can occur in a direct or indirect manner. Also isolated from this screen were genes involved in mismatch repair. Since defects in mismatch repair lead to an increase in homeologous recombination, these genes may lead to telomeric rearrangemets that allow survival of a *cdc13-Δ rad24-Δ* strain. Another class of mutants is those where the insertion is located within genes implicated in telomere maintenance. These include *EBS1*, *SGS1*, and *PIF1*. A number of other interesting mutants were isolated that did not appear to segregate into categories. These mutants include ORFs with unknown functions and independent candidates of known function. Among the unknown ORFs, we have partially characterized some of the unknown ORFs hit in our screen, and we called them NCI genes (negates *cdc13-Δ* induced lethality).

C. Determine the in vivo location of the protein, and investigate potential interactions with proteins involved in end protection (Characterization of secondary candidates): A candidate approach was first taken to identify proteins that, when deleted, promote viability in the absence of end protection. Candidates selected in this section were selected based on the criteria described for interesting candidates (see 2002 report).

The first candidate selected was ExoI. Mutants of ExoI were isolated which appeared to lead to enhanced growth of *rad24-Δ cdc13-Δ* cells. Based on this result we asked if loss of ExoI function would allow survival of a *cdc13-Δ* strain. Analysis of *cdc13-Δ exoI-Δ* cells indicated that abrogation of ExoI function is capable of promoting a partial bypass of *cdc13-Δ* lethality.

Another candidate that was studied in detail was Nam7 or Upf1, a protein involved in nonsense-mediated decay (NMD). Abrogation of Nam7 function in a *cdc13-Δ rad24-Δ* strain led to nearly wild type growth. We also observed that Rad24 function most be lost for Nam7 loss to have an effect in *cdc13-Δ* viability. This is different from the effect of loss of ExoI function, which can partially bypass *cdc13-Δ* even in the presence of Rad24. It has been reported that defects in Upf1, Upf2, and Upf3 can all lead to short telomeres and to overexpression of telomeric proteins such as Stn1 and Ten1 (Lew et al., 1998; Dahlseid et al., 2003), but the role of NMD in telomere maintenance is still under investigation.

Three novel genes recovered from the *NCI* screen were called *NCI1*, *NCI2*, and *NCI3*. These were re-tested for bypass of in a *cdc13-Δ rad24-Δ* strain by patch growth after serial dilutions (Figure 6). This method was used because it enables us to distinguish between background growth and growth advantages conferred by mutation of genes identified in the screen. *cdc13-Δ rad24-Δ* strains that have lost *NCI1* and *NCI3* function show an increase in colony formation after loss of the CDC13 covering plasmid (Figure 6a and c), while loss of NCI2 increases the frequency of "escaper" colonies after loss of a covering CDC13 plasmid (Figure 6b). Of these *NCI* genes, only *NCI1* has a telomere length defect (Figure 6d). *NCI1* and *NCI2* encode for unknown proteins, while *NCI3* maps to an intergenic region. Deletion of the genes flanking that region is underway to determine if the phenotype can be recapitulated.

Mutants in mismatch repair were also analyzed for patch growth after serial dilutions. Disruptions in *PMS1* and *MSH2* conferred a strong growth advantage to *cdc13-Δ rad24-Δ* strains (Figure 7 and data not shown). We hypothesize that the reason why these genes confer a growth advantage to the *cdc13-Δ rad24-Δ* strain is because the extended G-strand present in this strain is a substrate for homeologous recombination, which is upregulated when any of the genes mentioned here is mutated.

Another class of mutants that has been analyzed is that of genes that inhibit telomerase access to telomeres. This class of mutants was expected because a fusion between Cdc13 DBD and telomerase relieves the lethality of a *cdc13-Δ rad24-Δ* strain. *PIF1* is a gene identified in the screen that has a role in inhibiting telomerase access. When tested with the patch growth assay, a growth advantage was conferred to the *cdc13-Δ rad24-Δ* strain (Figure 8). We are currently investigating whether other candidates fall in this class of mutants.

D. Determine whether the isolated protein has exonuclease activity: No progress has yet been made in this area.

KEY RESEARCH ACCOMPLISHMENTS

- Elucidation of a specific molecular mechanism for the role of Cdc13 in end protection: that of a delivery molecule
- Identification of Stn1 (and possible associated molecules) as the primary effector of end protection in *S. cerevisiae*
- Identification of a 130-amino acid region of Cdc13 necessary and sufficient for telomerase recruitment to the telomere
- Reconstitution of a wild type telomere even in the absence of intact Cdc13 function
- Isolation of a set of mutants fulfilling the criteria for involvement in chromosome end processing in yeast
- Abrogation of Rad24 function imparts viability to cells that have lost the ability to protect the chromosome end
- The bypass of *cdc13-Δ* by loss of Rad24 function is only partial, and results in microcolony growth. Rare cells escape this fate and grow to wild type size colonies, and they appear to do so by a recombination-based method of telomere maintanance
- Abolishing Exo1 function led to microcolony growth of *cdc13-Δ RAD24* strains, suggesting that Exo1 actively plays a role in wild-type telomeric processing
- Loss of Nam7 function promotes WT growth in *cdc13-Δ rad24-Δ* cells
- Design and implementation of a large-scale genetic screen to identify proteins involved in processing of the chromosome termini has been completed. 544 primary candidates and 132 secondary candidates have been isolated in the screen. 72 of the secondary candidates have been cloned and partially categorized
- Characterization of the secondary genes is in process. At least three new genes (NCI) have been identified that confers growth to a *cdc13-Δ rad24-Δ* strain. Other categories that are being studied are: genes involved in mismatch repair, and genes that enhance telomerase access to telomeres.
- Description of physical interaction between Cdc13 and Est1 by yeast two hybrid

REPORTABLE OUTCOMES

Erin Pennock:

Presentations

Oral Presentations

Telomerase and Telomere Dynamics in Cancer and Aging Meeting, San Francisco, June 2000.

Department of Molecular and Human Genetics Annual Retreat, February 2001.

Cold Spring Harbor Laboratory: Telomeres and Telomerase, April 2001.

Graduate Student Symposium Platform Talk: Baylor College of Medicine, August 2001.

Posters

FASEB: Yeast Chromosome Structure, Replication, and Segregation, August 1998.

Department of Molecular and Human Genetics Annual Retreat poster presentations, February 1999, 2000.

FASEB: Yeast Chromosome Structure, Replication, and Segregation, August 2000.

Cold Spring Harbor Laboratory: Telomeres and Telomerase, April 2003 (presented by E. Mandell).

Publications

Pennock, E., Buckley, K. and Lundblad V. Cdc13 delivers separate complexes to the telomere for end protection and replication. (2001) Cell 104:387-96.

Pennock, E. Towards an understanding of end protection in *Saccharomyces cerevisiae.* Dissertation Thesis. July, 2002.

Joel Otero

Presentations

Posters

Cell and Molecular Biology Annual Retreat poster presentation, April 2003

Cold Spring Harbor Laboratory: Telomeres and Telomerase, April 2003.

Conclusions

Several important findings were made during the funding period of this grant, one of them being the essential function of Cdc13. Based on our results, it is known that Cdc13 main function is to deliver an end protection complex that consists of at least Stn1 and Ten1to the telomere. A region of approximately 130 amino acids near the N-terminus of Cdc13 was identified as the telomerase recruitment domain, and genetic and physical interactions between Cdc13 and Est1, a component of telomerase were observed. Based

on genetic interactions between Cdc13 and other telomeric proteins, a screen was designed that provided us with new mutants that have long telomeres. At least one new gene involved in telomere maintenance was discovered in this screen. Another screen was designed to find activities that process telomeres. This screen was based on the assumption that if a gene X that process telomeres is mutated, then Cdc13 would be dispensable for viability. Genes identified in this screen are being characterized and this is an ongoing project. Since we discovered that the role of Cdc13 is to recruit a complex composed of Stn1 and Ten1 to telomeres, I am in the process of biochemically characterizing these two proteins. I am also in the process of producing multiple sequence alignments with both Stn1 and Ten1 to identify important residues and to find homologs of these proteins in higher organisms. This has become possible thanks to the many sequences that are available in several databases. In summary, research funded by this grant led to several findings that led to a better knowledge of chromosome end protection in *S. cerevisiae*. Since telomere maintenance is important to maintain chromosomal integrity in humans, this research has implications in cancer research because loss of that integrity is a critical step in carcinogenesis.

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APPENDIX COVER SHEET

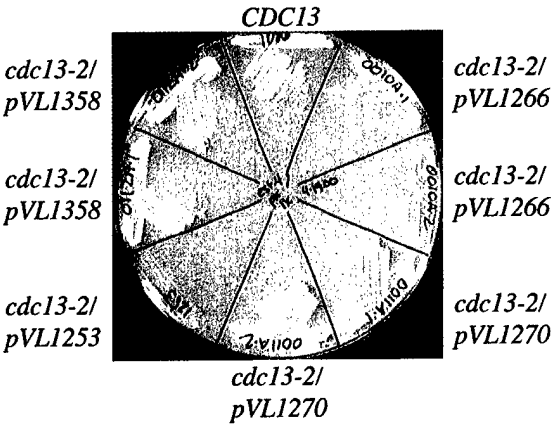
FIGURES 1-8

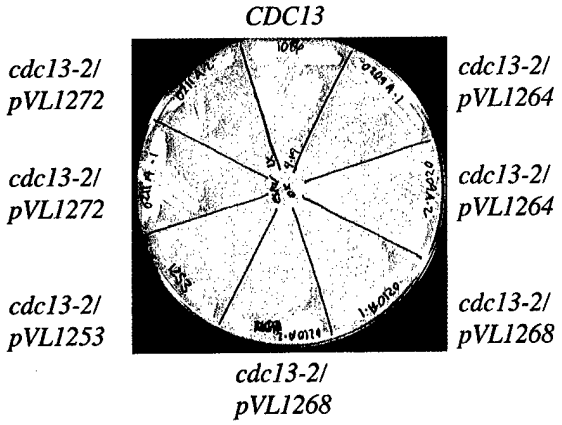
A

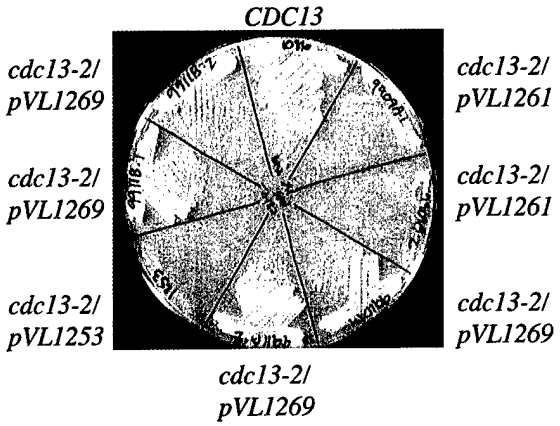
B

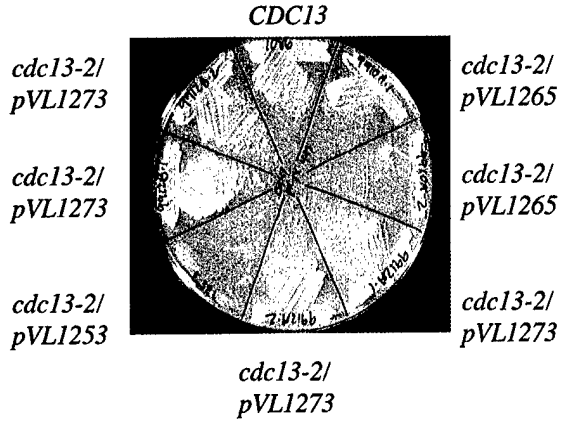
Figure 1. Stn1 retains its function when fused to the DBD_{CDC13} **(A)** *stn1-Δ* strains bearing the DBD_{CDC13}-*STN1* plasmid in high or low copy are capable of sustaining growth. Growth of *stn1-Δ*/pVL1045 *(STN1 CEN LEU2)*, *stn1-Δ*/pVL1136 *(DBD_{CDC13}-STN1 2μ LEU2)* and *stn1-Δ/* pVL1139 *(DBD_{CDC13}-STN1 CEN LEU2)* strains. Plasmids were introduced into a haploid *stn1-Δ*/pVL1046 (*STN1 URA3 CEN*) strain followed by subsequent eviction of pVL1046 on media containing 5-FOA; growth of the viable strains corresponds to ~25 generations after eviction of pVL1046. The DBD_{CDC13} -*STN1* constructs pictured here contain the previously defined DBD_{CDC13} (aa 452-693). **(B)** Telomeres in these strains are nearly wild-type. An *stn1-Δ/pSTN1* (pVL1045) strain is shown in lane1. Lanes 2-3 are *stn1-Δ/* DBD_{CDC13} *-STN1* (pVL1136). Strains were grown for ~35 generations following eviction of pVL1046 *(STN1 URA3 CEN)* on 5FOA media.

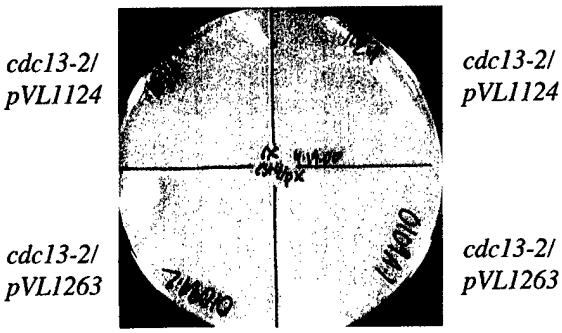
A



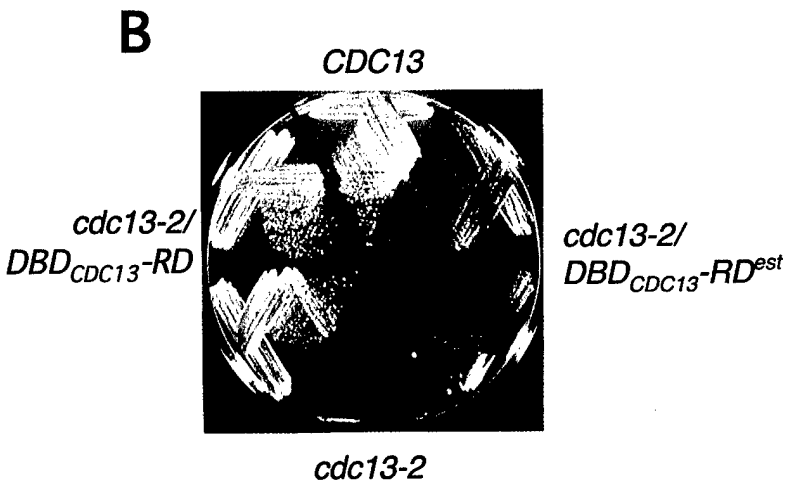








Plasmid	RD parameters	Rescue ability
1358	211-331	+++
1266	250-298	--
1270	190-298	++
1269	190-340	+++
1261	240-340	--
1263	240-331	--
1272	190-270	+
1268	250-270	--
1264	240-270	--
1273	211-340	+++
1265	250-340	--



1.CDC13+	
2.*cdc13^{est}*	35 gen
3.*cdc13^{est}*/pDBD-RD$_{190-340}$	75 gen
4.*cdc13^{est}*/pDBD- RD$_{211-340}$	75 gen
5.*cdc13^{est}*/pDBD- Rd$^{est}_{aa190-340}$	75 gen
6.*cdc13^{est}*	75 gen

Figure 2. Identification of the Recruitment Domain (RD) of Cdc13 **(A)** A DBD$_{CDC13}$-RD fusion protein complements the senescence phenotype of the *cdc13-2* mutant. A *cdc13-2* strain was transformed with the various plasmids containing DBD$_{CDC13}$-RD constructs, where the parameters of the putative RD differed from plasmid to plasmid. A DBD$_{CDC13}$ -STN1 plasmid (pVL1253), which cannot promote telomerase recruitment, was also transformed as a control for senescence. Following transformation, the strains were streaked onto the plates as shown, each streakout represents approximately 75 generations of growth since dissection from a recruitment-competent parent strain (YVL157). Each transformant was scored for the ability of the covering DBD$_{CDC13}$-RD plasmid to rescue senescence, and that data is summarized in the accompanying table. pVL1124 is a plasmid that contains the DBD$_{CDC13}$ only, and therefore serves as a second control for *cdc13-2* senescence. **(B)** Introduction of the *cdc13-2* mutation abolishes the ability of the DBD$_{CDC13}$-RD fusion protein to complement the senescence phenotype of the *cdc13-2* mutant. Growth after approximately 75 generations of a *cdc13-2* strain harboring plasmids expressing the wild-type Cdc13 protein (pVL1086), a vector control (pRS425), the DBD$_{CDC13}$-RD$_{aa190-340}$ fusion (pVL1269) or the DBD$_{CDC13}$-RD$^{est}_{aa190-340}$ version (pVL1368). **(C)** The DBD$_{CDC13}$-RD is capable of complementing the telomere length defect of *cdc13-2*, in addition to the senescence. A subset of the *cdc13-2/ DBD$_{CDC13}$-RD* strains that exhibited healthy growth were inoculated into 8mL of YPD and DNA was prepared from the resultant cultures, as well as an *est-* counterpart for a control. The number of generations each strain has been propagated since dissection from YVL157 is noted in the blot legend. The plasmids used in this experiment are as follows: pVL1086 (lane 1), pRS425 (lane 2), pVL1269 (both lanes 3), pVL1273 (lane 4), pVL1368 (lane 5), pRS425 (lane 6).

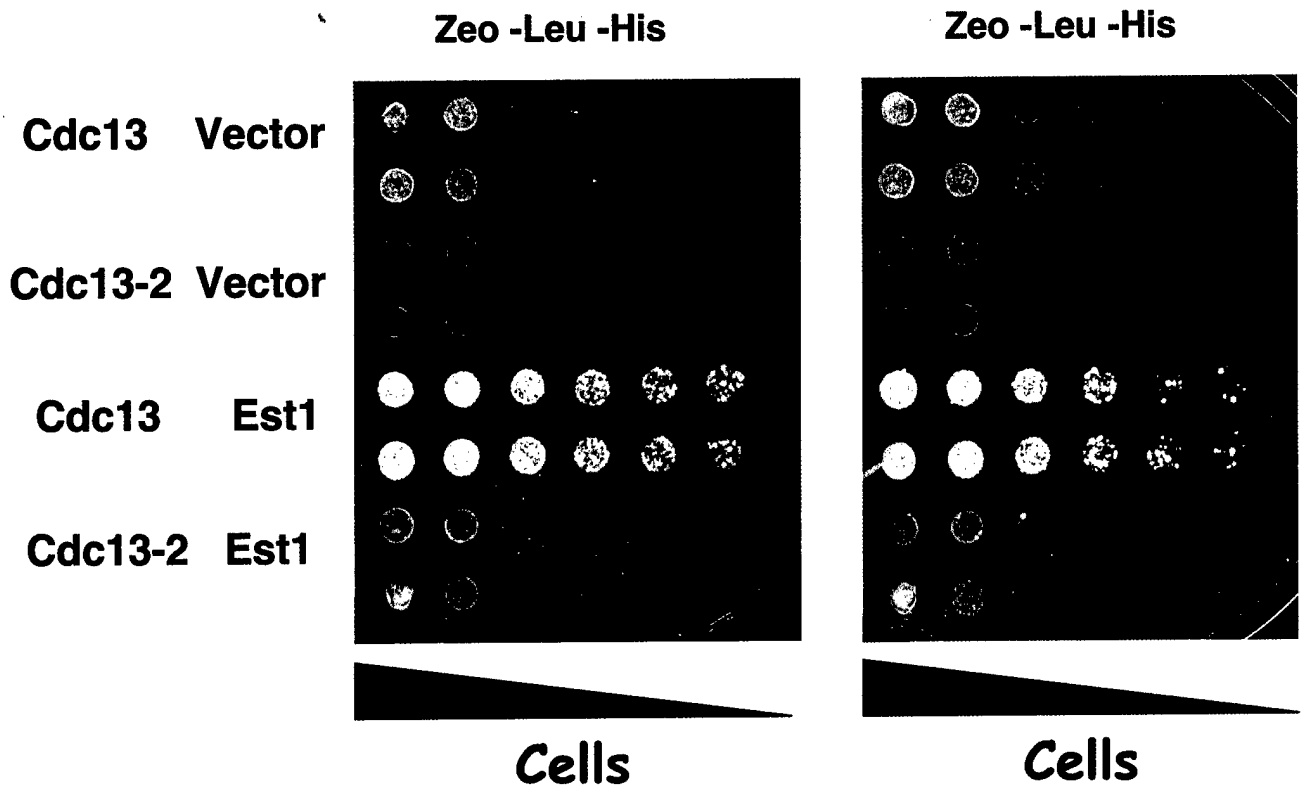


Figure 3. Yeast Two Hybrid Analysis of the Cdc13 and Cdc13-2 proteins with the telomerase component Est1. L40 cells were transformed with *CDC13 2μ ZEO*^{R} or *cdc13-2 2μ ZEO*^{R} plasmids (pVL2333 or pVL2334) and *EST1 2μ LEU* plasmid (pVL693) or empty vector (pACT2). Two different repeats of this experiment are shown here. Serial dilutions were plated on –Leu +Zeo –His media to monitor the expression of a *HIS3* reporter gene, which is under the control of the LexA upstream activator sequence (UAS). Cells transformed with Cdc13 plasmids and empty vector showed background levels of growth in this assay (rows 1-4), as did cells transformed with the Cdc13-2 and Est1 plasmids (rows 7-8). Only cells that were transformed with Cdc13 and Est1 show growth levels above background. This growth is not due to a difference in the density of cells plated, since we tested this in media that selects for both plasmids and cell density was similar for all transformants (not shown).

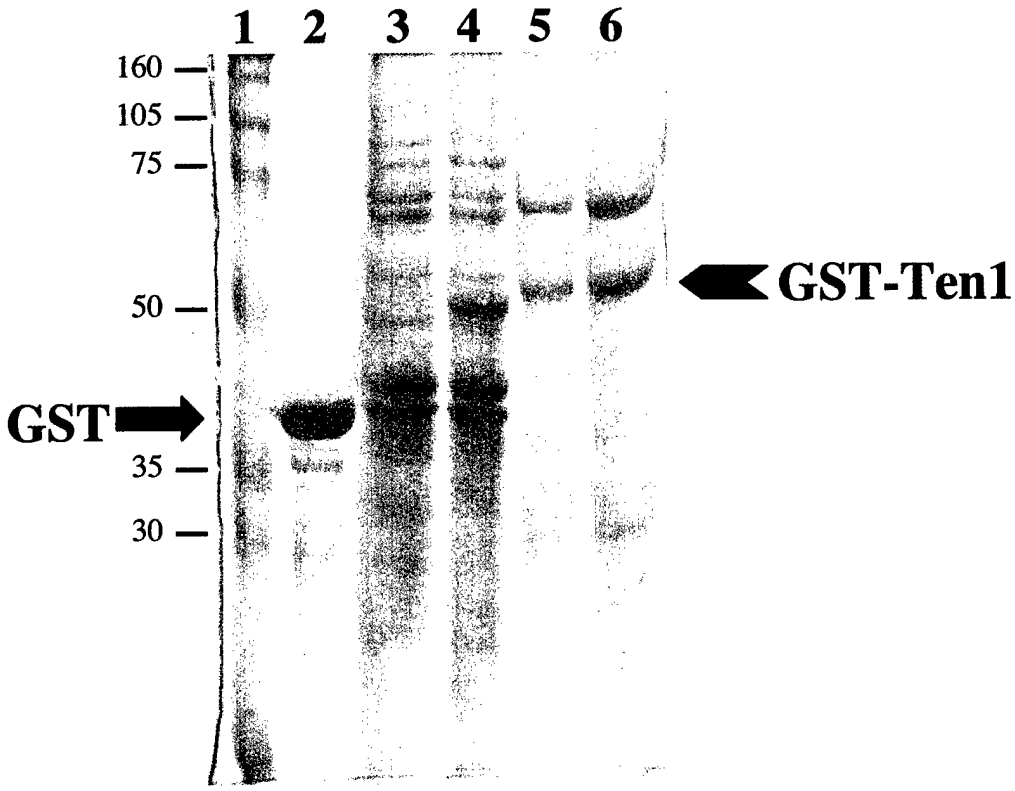


Figure 4. Purification of GST-Ten1. BL21 *E.coli* cells were transformed with a plasmid encoding Ten1 with a GST tag in its N-terminus. Liquid cultures of LB with kanamycin were grown at 37°C until the OD_{600} reached 0.6 and then were induced with 0.4mM IPTG and grown at room temperature for 3 hours. The tagged protein was then purified by batch method using Glutathione Sepharose 4B (Amersham). Lane 1 is the molecular weight marker, and lane 2 represents purified GST (control). A band of around 50 kD appears in the induced fraction (lane 4) that is not present in the uninduced fraction (lane 3). An enriched or partially purified preparation of GST-Ten1 is obtained after elution with glutathione buffer (lanes 5-6). The identity of the obtained band as GST-Ten1 was confirmed by immunoblotting with a GST antibody (not shown).

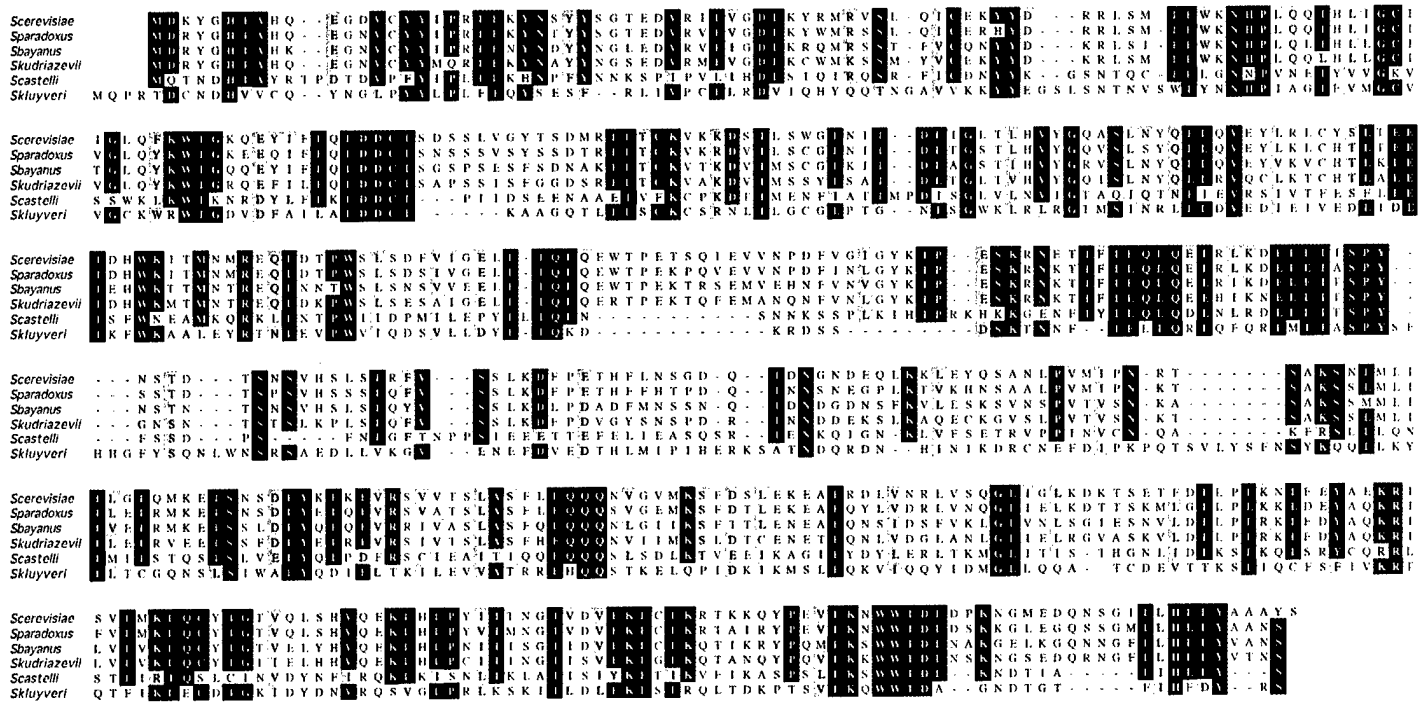
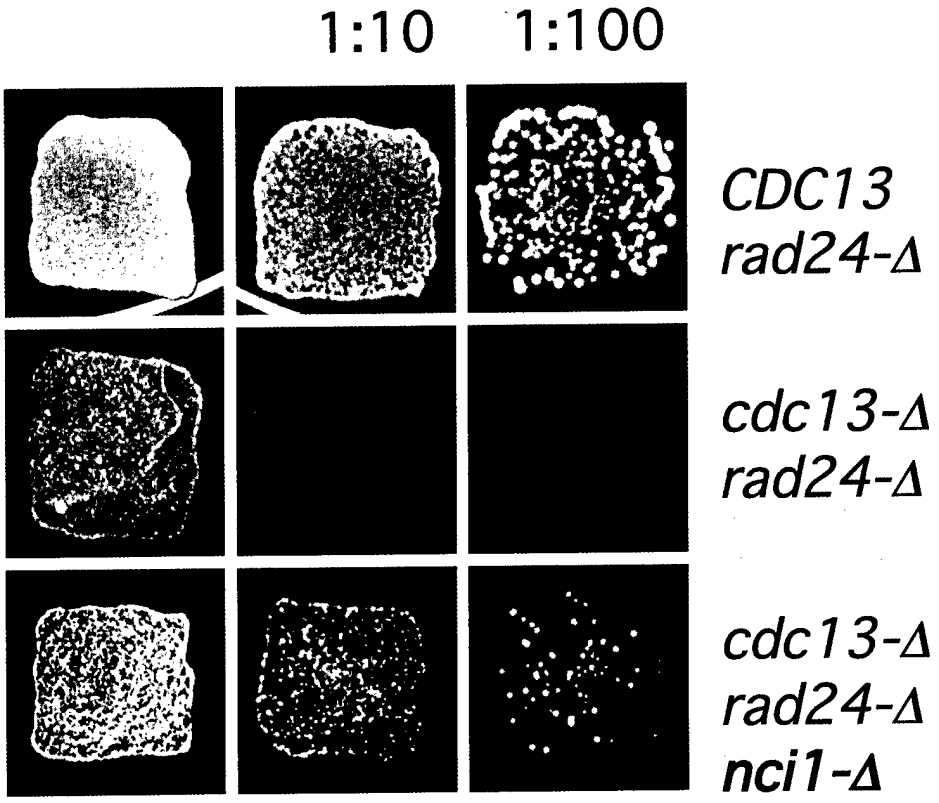


Figure 5. Multiple sequence alignment of Stn1 homologs from different *Saccharomyces* species. Amino acid sequences for Stn1 homologs from *S.bayanus*, *S.paradoxus*, and *S.castelli* were obtained from the Saccharomyces Genome Database (SGD), and the sequence from *S.kluyveri*, and *S.kudriazevii* was obtained from the Washington University's Genome Sequencing Center (GSC) database. The alignment was performed with the MacVector program. Identical residues are indicated by black squares, while similar residues are indicated as grey squares.

A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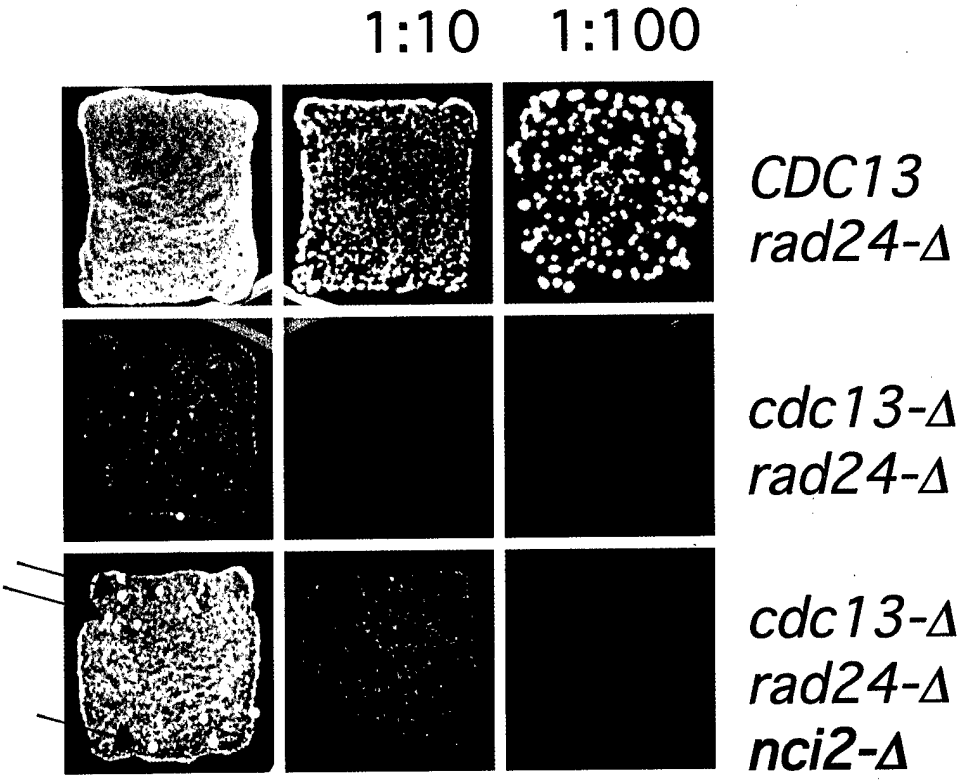
rad24-Δ
cdc13-Δ
rad24-Δ
nci1-Δ

B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rad24-Δ
nci2-Δ

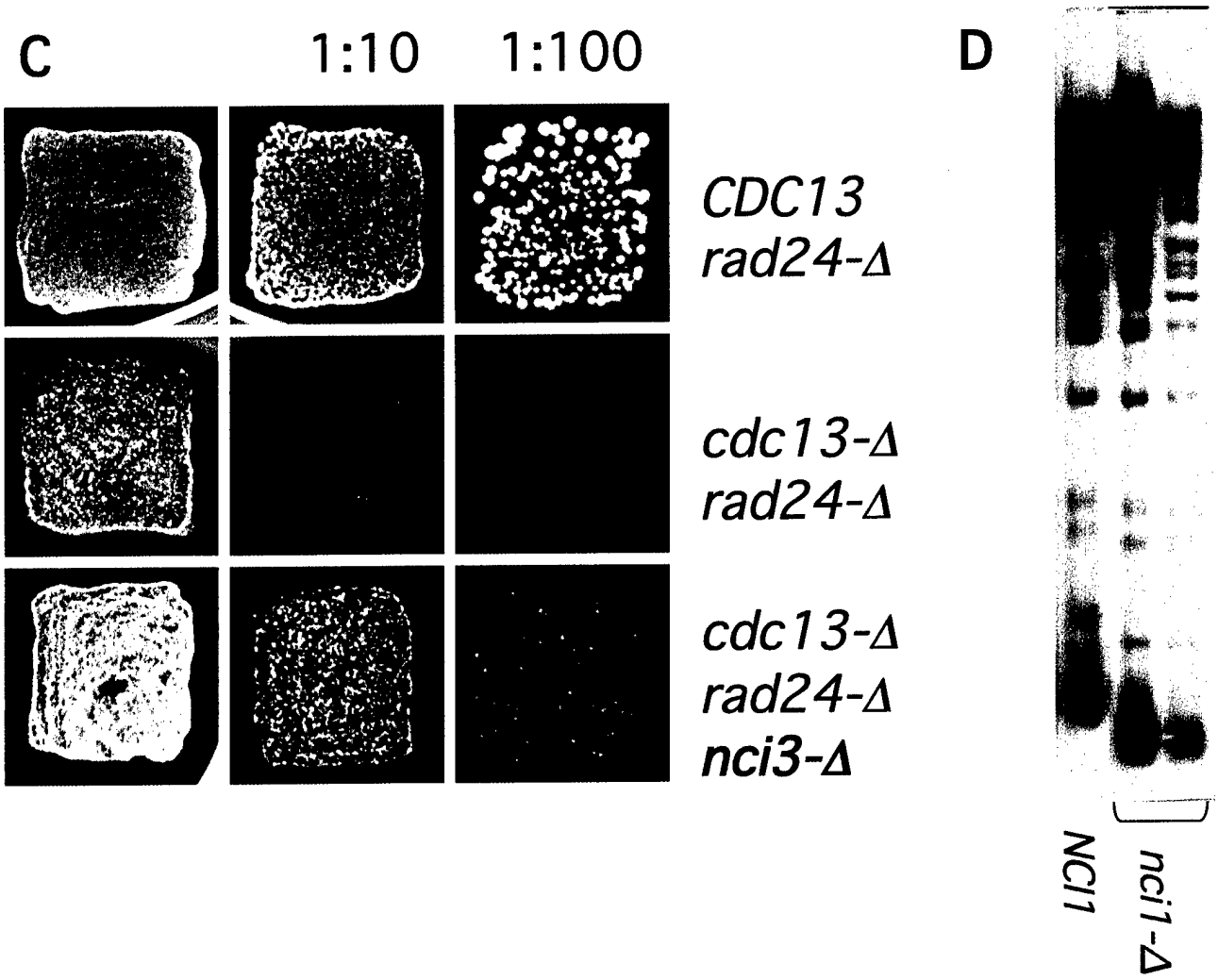

Figure 6. Three novel genes recovered from the NCI screen. Three of the genes found in the screen were subjected to a patch growth assay in which *cdc13-Δ rad24-Δ nci-Δ* cells were grown in liquid media and then spotted and spread in squares in -Leu 5-FOA media which will only allow growth of cells that have lost the covering *URA3-CDC13* plasmid. **(A)** Serial dilutions of *CDC13 rad24-Δ, cdc13-Δ rad24-Δ,* or *cdc13-Δ rad24-Δ nci1-Δ* cells were plated in -Leu 5-FOA after growth in liquid media. The last row shows that the absence of Nci1 confers a growth advantage to the *cdc13-Δ rad24-Δ* strain. **(B)** In the case of Nci2 colonies do not form as in cells lacking Nci1, but there is an increased frequency of "escaper" colonies (red arrows). **(C)** Loss of Nci3 has the same effect as loss of Nci1. **(D)** Telomeric Southern Blot of wild type cells (lane1) and *nci1-Δ* cells.

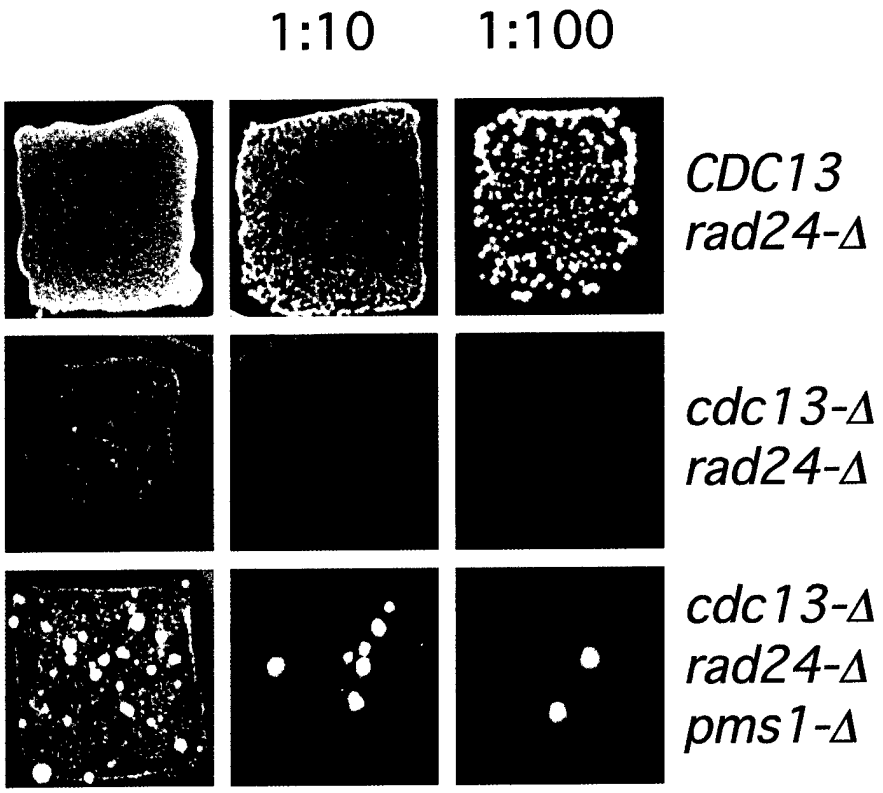

Figure 7. Mutants that enhance recombination. *cdc13-Δ rad24-Δ pms1-Δ* cells were analyzed as in Fig. 6. As the bottom row shows, a deletion in *PMS1* confers a NCI phenotype. Similar results were obtained for a *cdc13-Δ rad24-Δ msh2-Δ* strain (not shown). Other genes with roles in mismatch repair or recombination found in the screen that will be tested in a similar way.

Table 3: Occurrence of LOH in different areas of endometrial stromal neoplasms (ESN, ESS)

Case number	Diagnosis	DNA marker	Location of alteration
3	LESS	D5S107	ESS
3	LESS	D10S610	NE & NM
4	ESN	D3S1300	ESN
4	ESN	D10S610	NE
8	LESS	D3S1300	ESS
8	LESS	D5S107	ESS
9	LESS	D10S2491	NM
10	HESS	D10S185	ESS
10	HESS	D10S221	MCT
10	HESS	D10S185	MCT
13	HESS	D10S610	MCT
14	LESS	D14S267	MCT
15	ESN	D17S785	ESN
15	ESN	D14S267	MCT
15	ESN	D10S610	MCT & NM
17	LESS	D17S579	NSC
19	HESS	D10S221	MCT
20	LESS	D10S221	NE & NCE
20	LESS	D11S1311	NSC
21	LESS	WT1	ESS
22	LESS	D14S267	ESS
22	LESS	D11S1311	ESS

22	LESS	TP53	ESS
24	LESS	D10S185	ESS
24	LESS	D10S2491	ESS
27	LESS	D10S221	ESS

LESS: low grade ESS; HESS: high grade ESS; ESN: endometrial stromal nodule; MCT; myometrium close to the tumor; NE: normal endometrium (distant from the tumor); NM: normal myometrium (diatant from the tumor); NSC: normal stroma of the cervix; NCE: normal cervical epithelium.

Endometase in Human Breast Carcinomas, Selective Activation of Progelatinase B and Inhibition by Tissue Inhibitors of Metalloproteinases-2 and -4[1]

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Running title: Matrilysin-2 in human breast cancer

Key Words: Endometase/matrilysin-2/matrix metalloproteinase-26, pro-gelatinase B activation, tissue inhibitors of metalloproteinases, human breast cancer, cancer-specific expression.

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[3] **The abbreviations used are:** AIDH, atypical intraductal hyperplasia; DCIS, ductal carcinoma *in situ*; ECM, extracellular matrix; FN, fibronectin; IDC, infiltrating ductal carcinoma; IMA, integrated morphometry analysis; MMP-9, matrix metalloproteinase-9; MMP-26, matrix metalloproteinase-26; TIMP, tissue inhibitor of metalloproteinases.

ABSTRACT

Human endometase/matrilysin-2/matrix metalloproteinase-26 (MMP-26)-mediated pro-gelatinase B (MMP-9) activation promotes invasion of human prostate carcinoma cells (Zhao *et al.*, *J. Biol. Chem.*, 278: 15056-15064, 2003). We have also investigated the role of tissue inhibitors of metalloproteinases (TIMP-1, -2 and -4) in regulating the MMP-26-mediated activation of MMP-9 and the gene and protein expression patterns of MMP-26 in human breast cancer tissues. Enzyme kinetic studies showed that TIMP-1, -2 and -4 were potent inhibitors of MMP-26, with apparent K_i values of 4.7, 1.6, and 0.62 nM, respectively. TIMP-2 and -4 completely and TIMP-1 partially, in 10-fold molar concentration of MMP-26, blocked the activation of pro-MMP-9 by MMP-26. Higher levels of mRNA and protein for MMP-26 were detected in breast cancer cells than those of normal breast epithelial cells surrounding the carcinomas in human breast tissues by *in situ* hybridization and immunohistochemistry, and by reverse transcriptase-polymerase chain reactions using microdissected tissues. The expression levels of MMP-26 protein in ductal carcinomas *in situ* (DCIS) were significantly higher than those in infiltrating ductal carcinomas (IDC), atypical intraductal hyperplasia, and normal breast epithelia adjacent to DCIS and IDC. Double immunofluorescence labeling and confocal laser scanning microscopy revealed that MMP-26 was co-localized with MMP-9, TIMP-2 and -4, respectively, in DCIS. MMP-26 may play a more important role in the initiation of human breast carcinoma invasion from DCIS to IDC rather than at a later stage in IDC spreading, therefore, MMP inhibitors may have targets in earlier stages of cancer progression.

INTRODUCTION

Matrix metalloproteinases (MMPs) are believed to be associated with cancer-cell invasion and metastasis (1, 2). MMP-26 is a novel enzyme that was recently cloned and characterized by our group (3) and others (4-6). It has several structural features characteristic of MMPs, including a signal peptide, a propeptide domain, and a catalytic domain with a conserved zinc-binding motif (3-6). However, it has a unique "cysteine switch" sequence in the prodomain, PHCGVPD instead of the conserved PRCGXXD found in many other MMPs, which keeps the enzyme latent.

MMP-26 mRNA is primarily expressed in cancers of epithelial origin, such as endometrial carcinomas (3, 7), prostate carcinomas (7), lung carcinomas (7), their corresponding cell lines (3-6), and in a small number of normal adult tissues, such as the uterus (3, 5), placenta (4, 5) and kidney (6). We also reported that the levels of MMP-26 gene and protein expression are higher in a malignant choriocarcinoma cell line (JEG-3) than in normal human cytotrophoblast cells (8). Recently, we found that MMP-26 mRNA and proteins are highly expressed in human prostate carcinoma tissues and cell lines (9). The levels of MMP-26 protein in human prostate carcinomas from multiple patients were significantly higher than those in prostatitis, benign prostate hyperplasia and normal prostate tissues. MMP-26 and MMP-9 are also co-expressed in human prostate carcinoma tissues and highly invasive and metastatic androgen-repressed prostate cancer (ARCaP) cells. MMP-26 is capable of activating pro-MMP-9 by cleavage at the Ala (93)-Met (94) site of the pro-enzyme, and this activation facilitates the efficient cleavage of fibronectin (FN) and promotes the invasion of ARCaP cells across FN or type IV collagen (9). The activation is prolonged but persistent, which is consistent with the process of tumor cell invasion. These findings indicate that MMP-26-mediated pro-MMP-9 activation may be one biochemical mechanism contributing to human carcinoma cells invasion *in vivo*.

MMP activities are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs). Four mammalian TIMPs have been identified: TIMP-1 (10), TIMP-2 (11), TIMP-3 (12), and TIMP-4 (13). The hydrolytic activity of MMP-26 against synthetic peptides is blocked by TIMP-1, -2, and -4 (5, 6, 8), but the inhibitory potential of TIMP-1 is lower than that of TIMP-2 and TIMP-4 (5). TIMP-1 and TIMP-2 also inhibit the cleavage of denatured type I collagen (gelatin) by MMP-26 (6). TIMPs are expressed in human breast cancer cells (14-16). Here, we continue to explore the possible roles of MMP-26 and the coordination of MMP-26 with MMP-9, TIMP-2 and -4 in human breast carcinoma invasion.

In the present study, we showed that TIMP-2 and -4 completely and TIMP-1 partially inhibited the activation of pro-MMP-9 by MMP-26. Higher levels of MMP-26 mRNA and protein were detected in human breast carcinomas when compared to normal breast epithelia around the cacinomas. The expressions of MMP-26, MMP-9, TIMP-2 and TIMP-4 proteins in human breast ductal carcinomas *in situ* (DCIS) were significantly higher than those in infiltrating ductal carcinoma (IDC), atypical intraductal hyperplasia (AIDH) and normal breast epithelia around the DCIS and IDC. Furthermore, MMP-26 was co-localized with MMP-9, TIMP-2 and -4 in human breast DCIS. Our data suggest that MMP-26/-9 and TIMP-2/-4 may play an important role in the initiation of human breast carcinoma invasion from DCIS to IDC.

MATERIALS AND METHODS

Inhibition assays of MMP-26 by TIMP-1, -2, and –4. The quenched fluorescence peptide substrates, *Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2* and *Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2* were purchased from Calbiochem. The concentration of active MMP-26 was determined by active site titration with the tight-binding inhibitor GM-6001 as described

(17). GM6001 was the most potent inhibitor of MMP-26 tested, with a K_i^{app} of 0.36 nM (17). Because TIMPs are tight-binding and slow-binding inhibitors of MMPs, MMP-26 was incubated for 4 hours with TIMP-2 and –4, or 6 hours with TIMP-1, prior to the measurement of substrate hydrolysis to allow the enzyme and inhibitor to reach their binding equilibrium. The concentrations of TIMPs ranged from 0.2 to 60 nM. The assay was initiated by the addition of a substrate stock solution (4 µl) prepared in 1:1 water and dimethylsulfoxide to an enzyme-inhibitor assay buffer (196 µl) for a final concentration of 1 µM. The release of the fluorogenic cleavage product was monitored by measuring fluorescence (excitation and emission wavelengths at 328 and 393 nm, respectively) with a Perkin Elmer Luminescence Spectrophotometer LS 50B connected to a water bath with a temperature control. All kinetic experiments were conducted in 50 mM HEPES buffer containing 10 mM $CaCl_2$, 0.2 M NaCl, and 0.01% Brij-35. Fluorogenic peptide substrate assays were performed following the procedures we previously reported (17). To assess inhibition potency, the apparent inhibition constants (apparent K_i values) were determined by fitting the two trial data sets to the Morrison equation below (18) with non-linear regression.

$$\frac{v_i}{v_o} = 1 - \frac{\left([E]+[I]+K_i^{app}\right) - \sqrt{\left([E]+[I]+K_i^{app}\right)^2 - 4[E][I]}}{2[E]}$$

In this equation, v_i is the initial rate of MMP-26 catalysis in the presence of inhibitor and v_o is the initial rate without inhibitor. [E] and [I] are the initial or total enzyme and inhibitor concentrations, respectively, and K_i^{app} is the apparent inhibition constant.

Pro-MMP-9 activation by MMP-26 and inhibition of activation by TIMP-2 and TIMP-4. Zymography and silver staining were performed as previously reported (3, 7, 19, 20). Human fibroblast TIMP-1 and TIMP-2 were gifts from Dr. L. Jack Windsor (Indiana University, IN). Recombinant human TIMP-4 was purchased from R&D Systems, Inc. (Minneapolis, MN). MMP-26, pro-MMP-9 and active MMP-9 were purified in our laboratory (3, 21). The molar concentration ratios of TIMPs, MMP-26 and pro-MMP-9 were 10:1: 4. Two broad-spectrum metal chelators/metalloproteinase inhibitors; 1, 10-phenanthroline (OP) and ethylenediaminetetraacetic acid (EDTA) were used as controls. Briefly, MMP-26 was incubated in the absence or presence of different inhibitors (TIMPs, OP, and EDTA) in 30 µl of 1× HEPES buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, and 0.01% Brij-35) at room temperature (25 °C) for 4 hours. Pro-MMP-9 was then added and incubated at 37 °C for 20 hours. For zymography, aliquots of the reaction solution were removed and treated with a non-reducing sample buffer. MMP-9 activity was analyzed by zymography on 9% SDS (sodium dodecyl sulfate)-polyacrylamide gels containing 1% gelatin (22). For silver staining, aliquots were removed and treated with a reducing sample buffer and boiled for 5 minutes. Following electrophoresis on a SDS-polyacrylamide (9%) gel, the protein bands were visualized by silver staining (19).

Fibronectin cleavage assay. MMP-26, pro-MMP-9, MMP-26-activated MMP-9 and TIMPs were prepared as described above. Active MMP-9, which was purified from human neutrophils (21), was used as a positive control. Fibronectin (FN) was incubated with MMP-26, pro-MMP-9, active MMP-9, or MMP-26 plus pro-MMP-9 in the presence or absence of TIMP-2 or TIMP-4 in 1× HEPES buffer at 37 °C for 18 hours. The molar concentration ratios of MMP-26: pro-MMP-9: FN: TIMP were approximately 1:4:10:10. Aliquots were removed and treated with a reducing

sample buffer and boiled for 5 minutes. Samples were then loaded onto 9% polyacrylamide gels in the presence of SDS, electrophoresed, and subjected to silver staining (19).

Microdissection and RT-PCR assays. Under RNase-free conditions, human normal breast tissues and breast carcinomas were collected. 10 μm cryostat sections were prepared. The PixCell II LCM system was used to microdissect cells from H&E-stained frozen tissue sections. Amplitude and pulse duration of the PixCell II laser were adjusted to allow complete tissue capture using a 15 μm laser beam. The microdissecting time for each section was confined to 15 minutes to prevent RNA degradation. The collected samples included normal epithelia (NE), DCIS, IDC and normal epithelia around IDC. After microdissection of each specimen, the thermoplastic film-coated cap containing the captured cells was placed in a microtube and the total RNA was extracted using a micro-RNA isolation kit (Stratagene) according to the manufacturer's recommendations. The RNA was resuspended in 12 μl DEPC-treated water and DNase (RQ1 RNase-free DNase, Promega) prior to reverse transcriptase (RT)-PCR (Invitrogen Corporation, Carlsbad, California) according to the manufacturer's protocol and our previous report (9). The MMP-26 forward primer was 5'-CAGCTCGTCATCTTAAGAGTTAC-3'; the reverse primer was 5'-AGGCATGTCAGATGAACATTTTTC-3'; for β-actin (GI: 5016088) the forward primer was 5'- CGCGAGAAGATGACCCAGATCATG -3'; the reverse primer was 5'-AGGATCTTCATGAGGTAGTCAGT-3'. PCR reactions were performed using a Biometra Personal Cycler (Biometra, Germany) with 35 thermal cycles of 30 sec 94°C denaturing, 30 sec 55°C annealing, and 1 min 72°C elongation. The expected PCR product for MMP-26 is 750 bp, and for β-actin, 223bp.

***In situ* hybridization.** The full length MMP-26 sense gene and anti-sense gene were amplified in pCR 3.1 and purified as our previous report (9). The sense and antisense plasmids were

explained.